

Original Research Article

Nutrient feeding strategy determines the fate of Microalgal growth and carbon metabolizing enzyme system - A study with *Desmodesmus commuis* LUCC 002

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ABSTRACT

Keywords

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Biomass;
Zinc;
Copper.

The increasing applications of microalgae have led researchers worldwide to investigate a reliable, cost effective, and fast means of biomass production. In this study commercial, agricultural fertilizer was used for algal nutrients as an approach for reducing the production cost of biomass. To optimize biomass production, two different strategies of nutrient feeding were tested: [#1] supplying all of the nutrients required for the entire growth phase in one initial feeding; [#2] feeding the optimal quantity of nutrients at a regular interval as it was consumed by the algae. Between the two tested strategies the second method was found to be ideal in achieving maximum biomass while also enhancing the activity of the carbon metabolizing enzyme carbonic anhydrase (CA). Both of the systems, fed with different strategies, received equal amounts of nutrients. The results clearly show that the feeding strategy is the only responsible factor for inducing the carbon metabolizing system. Esterase isozyme profiling was studied to understand the stress response exerted by the organism while growing in the different conditions created by the feeding strategies. It was clear that the first feeding method stressed the *Desmodesmus commuis* LUCC 002, this was evident from the stunted growth comparing to the second method.

Introduction

Microalgae tend to respond variably on different nutrient variations, either in the situation of deprivation or over enrichment. This characteristic feature made researchers subjugate the metabolism of microalgae to impel

various bioproducts. All the algal media formulations contain excess nutrients than the optimal requirements, in most of the algal cultivation system unused residual nutrients remains in the medium. After harvesting the biomass reusing the

previously cultivated water for the consecutive fresh batch are also in practice. Based on the desired bioproduct and its application algal cultivation mode and method will be chosen. In case of bioenergy production, as the final product going to be applied on machines hygienic practices are not appreciated while cultivation. Whereas in case of utilizing the biomass as a feed source, care should be taken at all levels. The present work aimed to utilize the biomass for bioenergy production. Since the notion of the present experimentation is to enhance the biomass and lipid production, it is essential to maintain the cultivation system with optimal nutrient content. It is renowned that, microalgae triggers lipid accumulation at the nutrient deprived conditions (Ratha *et al.*, 2013). The percentage of lipid content on the biomass determines its bioenergy yielding capacity. Hence, once the cultivation system reaches the maximum growth, curtailing the nutrient concentration at a particular growth phase is important to make the algae accumulate lipid, also this venture enables to save time in the production process. Based on this principle two different strategies of nutrient addition were tested for the purpose of identifying a fastest means of biomass production, and to direct the algal physiology in acquiring desired bio-product.

Usage of agricultural fertilizers as algal nutrients are in practice, as well it yields good growth (Pacheco-vega, 2009) and replenishes the algal nutritional requirement and are easily available, further cost effective. Nitrogen, carbon, and phosphorus are the nutrient elements needed in greatest quantity by microalgae, carbon and nitrogen metabolism are closely linked. Under nitrogen deficiency, CO₂-concentrating mechanisms are

usually induced, which improves the nitrogen use efficiency of microalgal cells and allows the cells to maintain reasonable rates of CO₂ fixation with less investment of nitrogen in Rubisco (Beardall *et al.*, 1991). Therefore, on the anticipation of modulating the carbon capturing ability nutrient level was maintained marginal to the organism's requirement so as to induce the carbon metabolizing system. This study is the lead optimization for outdoor cultivation, principally - this investigation was carried out for reproducing the observed lab scale experimental outcome.

Materials and Methods

Experimental organism and culture conditions

The microalgae, *Desmodesmus communis* LUC 002 was obtained from the culture collection of Center for Bioenergy, Lincoln University in Missouri, USA. The organism was maintained and grown in 500 ml Erlenmeyer flasks containing 200 ml of synthetic BG 11 medium (Stanier *et al.*, 1971). Experimental cultures were incubated at 25 ± 2 °C, in a shaking platform with 120 rpm, 14:10 h light–dark cycle, with illumination of $27 \mu\text{E m}^{-2} \text{s}^{-1}$ under cool white fluorescent lights.

Experimental setup

Mid log phase culture grown in the laboratory at the above mentioned conditions was harvested by centrifugation at 4000rpm. The pellet was washed with distilled water and resuspended in 40 liters aquarium tanks. The initial inoculum was adjusted evenly (0.1 OD) in all the replicate tanks by dissolving the harvested thick slurry of biomass, and the final volume of the tanks was made to 10 liters. For agitation, aquarium tank (water

circulation) pump - Aqueon, Italy, with the flow rate of 500 gallons h⁻¹ was used. Commercial fertilizer with the chemical composition as represented in table 1, was used as a nutrient source and the feeding strategy was maintained as follows - in two replicate tanks of each system.

Feeding strategy #1 supplying nutrients altogether required for the entire growth phase, i.e., 7.4 grams of fertilizer in 10 liters.

Feeding strategy #2 feeding 740mg for 10 liters culture at the interval every three days.

The feeding concentration (74mg/L) was selected based on the optimal growth requirement of *Desmodesmus communis* LUCC 002 (data not shown). The aquarium tanks were maintained in green house provided with controlled temperature (25°C ± 2°C).

Biomass estimation and nutrient feeding

Every three days water level was maintained against evaporation and 15 ml of triplicate samples were obtained for growth estimation from each replicate tank. Once after sample collection 740 mg of fertilizer were added to the (strategy #2) tanks. The samples were filtered using 4.7 cm Whatman GF/C filters and dried at 80 °C overnight, and the mass of the dried biomass was measured gravimetrically. The results presented are the means of three independent experiments. Sample variability is given as the standard deviation of the mean.

Whole Cell Protein Preparation and Native PAGE

To study the response of carbonic anhydrase and esterase, cultures from the

tanks were harvested by centrifugation at 6000 rpm for 10 minutes. Then the thoroughly washed algal pellets were homogenized with an extraction buffer (62.5 mM Tris-Cl, pH 6.8) and the whole cell protein was extracted using 6800 freezer mill. Total soluble proteins, which served as the enzyme source, were obtained after three centrifugations, each for 20 minutes at 12000 rpm at 4°C. All the protein preparations were made inside an ice bath. Electrophoresis was carried out at 4 ± 1°C with 1.5 mm polyacrylamide gels in a Tris-glycine buffer (pH 8.3) under standard native conditions (Laemmli, 1970). A uniform amount (75 µg) of protein, estimated by Lowry et al., (Lowry *et al.*, 1951) was loaded with the sample buffer, devoid of sodium dodecyl sulfate and β-mercaptoethanol. Samples were then electrophoresed at 50 V through the stacking gel (5%) and at 100 V through the resolving gel (8%). Gel images were scanned by a Canon scanner (Canoscan LIDE210), and the enzyme activity profiles were analyzed by software provided with the gel documentation system (GeneTools, Syngene).

Activity staining of esterase (EC 3.1.1.1.)

Activity staining for esterases was performed by placing the gel in 100 ml of phosphate buffer (100 mM, pH 6.2) containing 50 mg α-naphthyl acetate, 50 mg β-naphthyl acetate and 100 mg fast blue RR salt. The gel was incubated at 25°C for one hour to develop black, red, or magenta color bands (Palanisami and Lakshmanan, 2010). Both the substrates (α-naphthyl acetate and β-naphthyl acetate) were dissolved quickly in 1 ml of acetone and mixed with the buffer solution just before the gel was transferred.

Activity staining of carbonic anhydrase (EC 4.2.1.1.)

The gel was incubated for 15 min in acetazolamide [carbonic anhydrase inhibitor] (10mg in 0.1 M Phosphate buffer, pH 7.2), then the activity staining for carbonic anhydrase was performed by placing the gel in 100 ml of 0.1 M Phosphate buffer, pH 7.2, α - Naphthyl acetate (dissolved in 20 mg of a few drops of acetone) and Fast Blue RR salt 50 mg. Incubate the gel in staining solution in the dark at 37°C until red bands appear. Wash the gel with water and fix in 7% acetic acid (Gennedy, 2003).

Result and Discussion

Growth variation of *D. communis* while growing on different feeding strategies

Biomass production and lipid accumulation are limited by a variety of factors, of which nutrients play key roles (Roleda *et al.*, 2013). Simultaneous biomass production and lipid accumulation is not a feasible and easy process, because lipid accumulation starts mostly at nutrient deprived conditions. It could be precise if the process has been segregated into two perspectives, acquiring maximum growth and then inducing the lipid accumulation. In the present study, the second method of nutrient feeding (strategy#2) i.e., adding nutrients as of the algae consumes found best than the method of adding nutrients at once (strategy#1) (Figure 1). Table 1 shows the quantity of nutrients used in both the feeding methods, the net nutrient quantity fed on both the system are same excepting the feeding intervals. It has to be considered that, the chemical composition of the used fertilizer imparted 370 μ g/L of copper and zinc to the medium.

Role of zinc and copper in the algal growth

Zinc is a well-known essential micronutrient for normal growth of algae. Its deficiency leads to poor growth and low dry weight (Omar, 2002). The growth response and tolerance of different species of algae to Zn have been reported by (Whitton, 1970), showed that algal growth would be stimulated by lower concentrations of zinc and totally inhibited by higher concentrations. Moreover, higher concentrations of Zn decrease the cell division, motility, total chlorophyll content, carotenoids:chlorophyll ratio and ATPase activity (Omar, 2002). Carbonic anhydrase (CA) is a zinc metalloenzyme that catalyzes the reversible reactions of CO₂ and water: $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}^+ + \text{HCO}_3^-$. Reports rendering evidence that higher concentration of zinc in the growth medium hinder cell division, which was evident from Figure 1. Both the feeding system was started with equal inoculams, after three days of growth the difference was distinct in strategy #1. Strategy #2 was started with tenfold nutrient lesser than strategy #1, the initial higher concentration of zinc might have imparted stress in strategy #1 and resulted in stunted growth. It was clear from the observed results that, *Desmodesmus communis* Lucc 002 can able to withstand the effect and grow on 370 μ g/L zinc. Whereas in strategy #2 as the addition of nutrients was step by step at every three days, this time duration made the organism to acclimatize, adapt and continue growth.

Copper is essential for various cellular process, as like the same physiology of zinc copper as well act as a growth stimulant in low concentrations and toxic at higher concentrations. Copper withstanding capability varies to different

Table.1 Chemical composition of the fertilizer used as algal nutrients – representing the concentrations of individual elements when 74mg dissolved in 1 L.

Chemical composition	74 mg/L of fertilizer holds (mg)	740 mg/L of fertilizer hold (mg)
Nitrate nitrogen	2.13	21.33
Ammonical nitrogen	0.74	7.4
Urea nitrogen	14.9	149.03
Available Phosphate[P2O5]	5.92	59.25
Soluble potash	11.85	118.51
sulfur[combined sulfur]	0.74	7.4
Boron	0.01	0.14
Copper [Chelated copper]	0.037	0.37
Iron [Chelated iron]	0.07	0.74
Manganese [chelated manganese]	0.03	0.37
Molybdenum	0.0007	0.007
Zinc [Chelated zinc]	0.037	0.37

Figure.1 Growth difference of *Desmodesmus communis* Lucc 002 upon supplementing with 740mg/L fertilizer at once and adding 74mg/L at every three days of interval

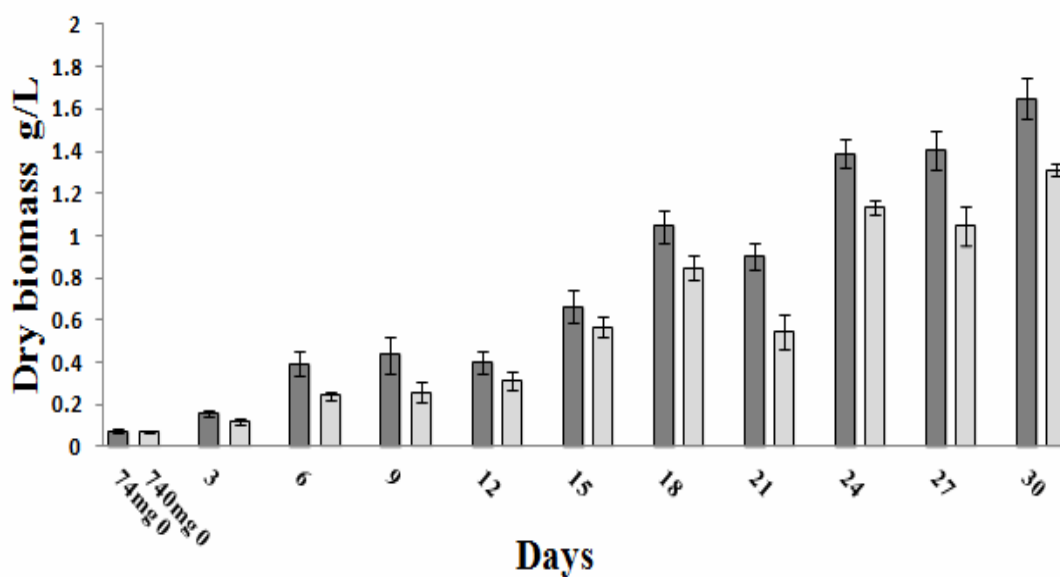
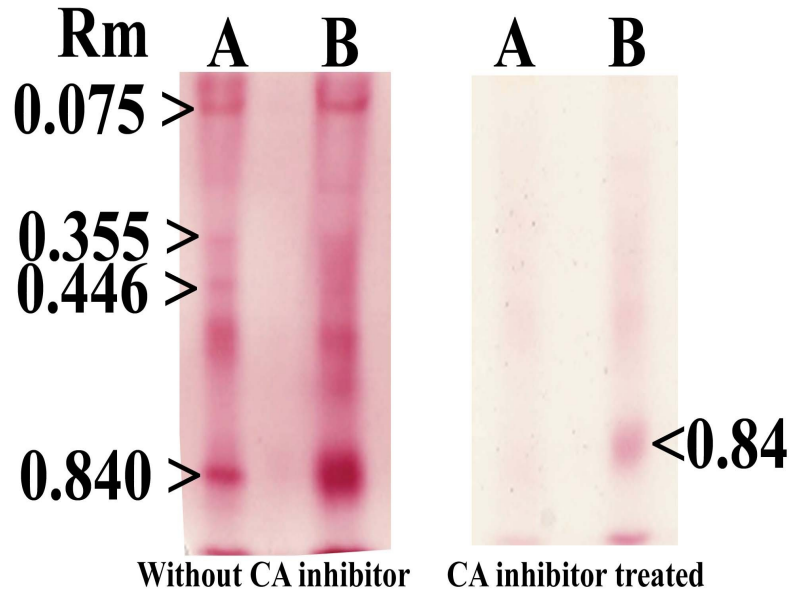


Figure. 2 Activity staining of carbonic anhydrase and esterase. Carbonic anhydrase (CA) activity was visualized without CA inhibitor, to subtract the substrate cross reactivity - esterase zymogram was visualized after treating with carbonic anhydrase inhibitor (A = 74mg/L; B = 740mg/L), [Rm = relative mobility].



taxonomical class of microalgae (*Debelius et al.*, 2009), from the obtained results it is apparent that *Desmodesmus communis* LUCC 002 can able to withstand acute and chronic type of metal exposure.

Microalgae are sensitive indicators of environmental change and, as the basis of most freshwater and marine ecosystems, are widely used in the assessment of risk and development of environmental regulations for metals (Levy *et al.*, 2007). Though the experimental species having the capability of withstanding the concentration of metals contain in the nutrients used, understanding its stress response paves way to improvise the biomass yield further. Activity staining of esterase was studied for a dual purpose, to confirm the cross reactivity of the substrate used in the staining of carbonic anhydrase and to evaluate and compare the stress response on both the feeding strategies. Esterase is one of the

biomarker reveals the physiological status of microalgae at metal stress (Palanisami and Lakshmanan, 2010). Feeding strategy #1 imparted stress to the organism which was evident from the increased expression of esterase located at Rm 0.84 on the zymogram visualized after treating with CA inhibitor. Strategy #2 triggered the activity of carbonic anhydrase; which was clearly evident by the expression of two new isoforms located at Rm 0.355 and 0.446 (Figure 2). Carbonic anhydrase contains zinc as prosthetic group, this new isoform expression suggest a phenomenon that, gradual addition of zinc improvise the carbon metabolizing system rather acting as toxicant. All the growth conditions maintained identical for both the feeding systems, the only difference existed is the nutrient feeding strategy. The revealed difference in the expression of new CA isoform clearly attests the principle relies only on the feeding strategy. Further narrowing down the concentration of

fertilizer and altering the sampling time length are in progress to standardize a perfect nutrient concentration and duration to attain maximum biomass in the shortest time. In addition, the results confirms the conception of (Beardall *et al.*, 1991), maintaining minimal nutrient especially nitrogen-enhances the carbonic anhydrase.

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